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Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells

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ABSTRACT **Curcumin, a dietary pigment responsible for the yellow color of curry, is a potent inhibitor of tumor promotion by phorbol esters. Functional activation of transcriptional factor c-Jun/AP-1 is believed to play an important role in signal transduction of phorbol 12-myristate 13-acetate-induced tumor promotion. Suppression of the c-Jun/AP-1 activation by curcumin is observed in mouse fibroblast cells. *In vitro* experiments indicate that inhibition of c-Jun/AP-1 binding to its cognate motif by curcumin may be responsible for the inhibition of c-Jun/AP-1-mediated gene expression. These findings show that the effect of curcumin on phorbol 12-myristate 13-acetate-induced inflammation/tumor promotion could be studied at the molecular level.**

Elevated expression of genes transcriptionally induced by phorbol 12-myristate 13-acetate (PMA) is among the events required for tumor promotion (1, 2). Analysis of these genes reveals the highly conserved motif 5′-TGASTCAG-3′ (where S is cytosine or guanine), conferring PMA inducibility (3, 4). This PMA-responsive element, referred to as TRE, also serves as the binding site for the AP-1 family of transcription factors. The protooncogene product c-Jun/AP-1 represents one member of the AP-1 proteins. Enhanced binding of c-Jun/AP-1 to the TRE in PMA-stimulated cells has been associated with the increased transcription of different responsive genes (5–7). On the other hand, glucocorticoid hormones inhibit phorbol ester tumor promotion and inflammation and drastically decrease collagenase gene expression (8–10). A TRE site within the collagenase promoter was found responsible for mediating the inducibility by PMA and inflammatory agents. The mechanism of interference is from repression of c-Jun/AP-1 activity by the glucocorticoid receptor. Therefore, activation of c-Jun/AP-1 is probably crucial in transmitting the tumor-promoting signals from the extracellular environment to the nuclear transcriptional machinery.

Curcumin, a dietary pigment responsible for the yellow color of curry, was reported to efficiently inhibit 98% of the PMA-induced tumor promotion on mouse skin (11). However, the molecular mechanisms of this inhibition are completely unknown. To study the mechanisms of the curcumin effect on PMA, we examined c-*jun* gene expression. The c-*jun* gene is positively autoregulated by its product c-Jun/AP-1 (6). When cells are treated with PMA, c-Jun/AP-1 activity is regulated at two levels. (*i*) An immediate post-translational event leads to increased DNA-binding activity of preexisting c-Jun/AP-1 protein. (*ii*) The activated preexisting c-Jun/AP-1 protein then binds to the TRE site in c-*jun* promoter, activating transcription. In this study, we show that suppression of the PMA-induced c-jun mRNA correlates with the amount of curcumin applied to mouse fibroblast cells. The increase in TRE-binding activity of nuclear extract after PMA treatment can be blocked by curcumin. The reduction of PMA-induced c-jun mRNA seems to be from attenuation of c-Jun/AP-1 TRE-binding activity. We also observed that curcumin repressed the expression of c-Jun/AP-1-responsive simian virus 40 (SV40)–(CAT) chloramphenicol acetyltransferase chimera. These results suggest that the suppression of TRE-binding activity is a mechanism for terminating the elevated expression of genes transcriptionally induced by PMA.

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MATERIALS AND METHODS

Chemicals. PMA was purchased from Sigma, and curcumin was obtained from Merck. Both chemicals were dissolved in absolute ethanol.

Cell Culture. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum/antibiotics (penicillin at 100 units/ml and streptomycin at 50 μg/ml)/2 mM glutamine. Confluent cells were serum-starved for 36–48 hr before different treatments, as indicated.

Probes. A *Pst* I–*Eco*RI fragment of 3′ c-*jun*-coding sequence (12), a 1-kilobase (kb) *Pst* I insert of pfos-1 (13), and the pAc18.1 fragment of rat β-actin (14) were random-primer-labeled and used as probes in Northern (RNA) blot and dot hybridization analysis. A synthetic, double-stranded oligodeoxynucleotide containing a TRE site, i.e.,

5′–GATCGTGACTCAGCGCG–3′
3′–CACTGAGTCGCGCCTAG–5′,

was ^{32}P-end-labeled by polynucleotide kinase in gel-retardation assay.

Northern Blot Analysis. Total cellular RNA was isolated, as described (15). Samples (15 μg) of RNA were separated on 1% agarose/formaldehyde gels and transferred onto nitrocellulose filters. Hybridization was for 12 hr at 42°C in hybridization buffer [50% (vol/vol) formamide/1 M NaCl/1% SDS/10% (wt/vol) dextran sulfate/5× Denhardt's solution/denatured salmon sperm DNA at 75 μg/ml]. Filters were extensively washed in 0.1× SSPE (1× SSPE = 150 mM NaCl/10 mM Na_xPO_4/1 mM EDTA, pH 7.4)/1% SDS at 52°C. After autoradiography, filters were boiled for 3 min in solution (10 mM Tris chloride/10 mM EDTA, pH 8.0) to remove old probes and were then reprobed with β-actin as an internal control.

Dot Hybridization. Five micrograms of RNA samples was heat-denatured and spotted onto the nitrocellulose filters. Hybridization and wash were done as described in Northern blot analysis. Finally, filters were cut and quantitated by liquid scintillation counting.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; TRE, PMA-responsive element; SV40, simian virus 40.

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Nuclear Extract. Nuclei were isolated according to the method of Greenberg and Ziff (16). Furthermore, crude nuclear extracts were prepared by resuspending nuclei in NE buffer [20 mM Tris chloride, pH 7.9/20% (vol/vol) glycerol/100 mM KCl/0.2 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/0.5 mM dithiothreitol] with a Kontes glass Dounce homogenizer (15 strokes with a type B pestle). After centrifugation at 2000 × *g* for 6 min at 4°C, the crude nuclear extract was saved from the pellet fraction.

Gel-Retardation Assay. Crude nuclear extracts (2 μg) were mixed with a ^{32}P-labeled double-stranded oligodeoxynucleotide containing a TRE site (0.5 ng) in 20 μl of 0.6× NE buffer. The entire reaction mixture was incubated at 30°C for 15 min and then loaded onto a 4% polyacrylamide gel. Electrophoresis was done at 35 mA in running buffer (50 mM Tris chloride, pH 8.5/0.4 M glycine/2.1 mM EDTA). The gels were dried and autoradiographed with intensifying screens at −75°C.

Transient Expression Assay. Cells were transfected by the calcium phosphate coprecipitation technique and exposed to the precipitate for 12 hr. Fresh Dulbecco's modified Eagle's medium/0.5% fetal calf serum was added after 3-min glycerol shock; dishes were treated with PMA at 50 ng/ml with or without 10 μM curcumin after 36 hr and were harvested 12 hr later. CAT activity was determined as described by Gorman *et al.* (17) and was measured by liquid scintillation counting of ^{14}C spots on TLC plates. A plasmid containing the Rous sarcoma virus long terminal repeat linked to the β-galactosidase gene was cotransfected to monitor the efficiency of transfection.

RESULTS

Curcumin Reduces the PMA-Induced c-jun mRNA Level. To examine the effect of curcumin on c-*jun* gene expression, Northern blot hybridization was used to analyze the amount of c-jun mRNA in NIH 3T3 cells treated with PMA with or without curcumin. Total RNA from confluent (quiescent) cells stimulated with PMA at 50 ng/ml was isolated at different times and analyzed by Northern blot hybridization (Fig. 1*a*). Relative abundance of c-jun mRNA was further quantitated by liquid scintillation counting of the excised spots of dot hybridization. The c-jun mRNA level of quiescent cells increased steadily up to 45 min after PMA addition and reached maximum at 60 min. This induction profile differs from that of c-*fos*, which peaked at 30 min. Thus, the

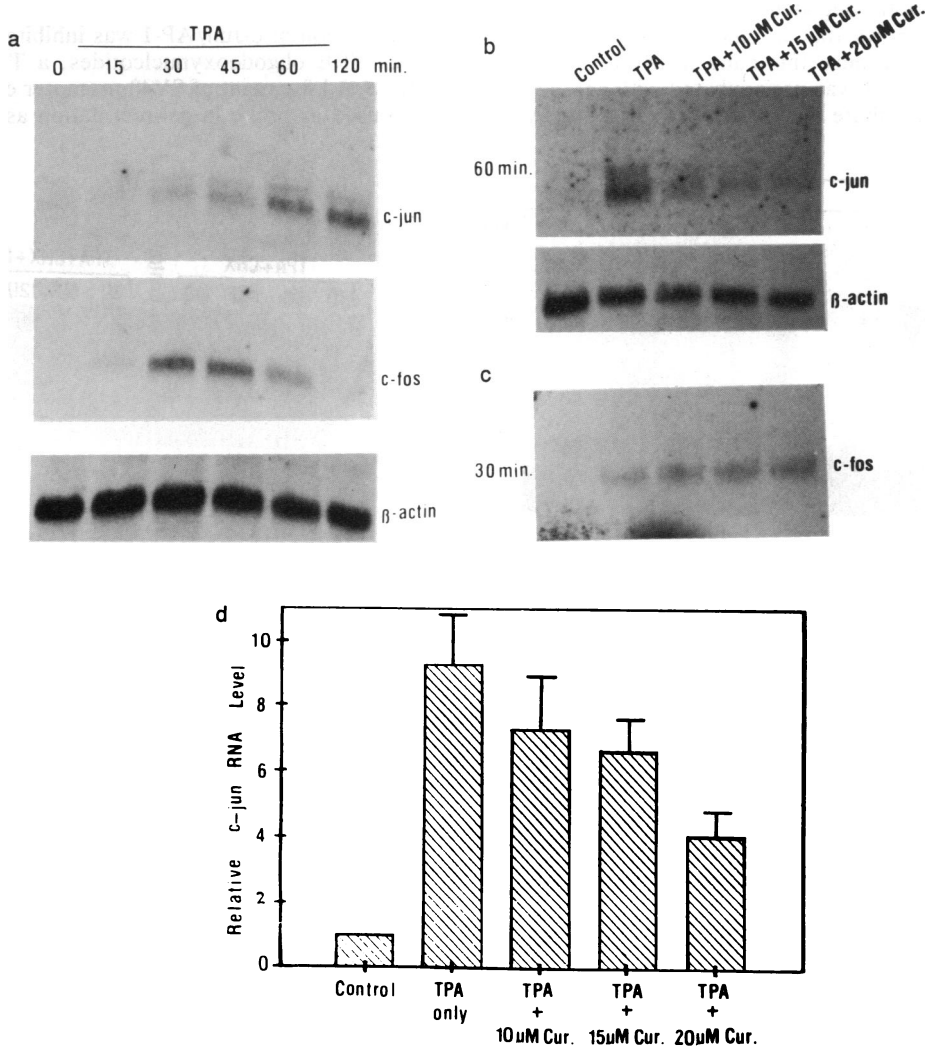

FIG. 1. (*a*) Northern blot analysis of c-jun and c-fos mRNA from quiescent cells treated with PMA (TPA) at 50 ng/ml for the indicated times. (*b*) Northern blot analysis of c-jun mRNA from quiescent cells and cells treated with PMA (TPA) for 60 min with or without curcumin (Cur.) at 10, 15, or 20 μM. (*c*) Analysis of c-fos mRNA after treatments (same as *b*) for 30 min. (*d*) Dot hybridization analysis to quantitate relative c-jun mRNA levels from cells treated as in *b*. The abundance of c-jun mRNA from different treatments was normalized to β-actin mRNA, and the relative levels of c-jun mRNA were obtained by normalizing each abundance to control. The result represents a mean of three independent experiments.

maximal increase of c-jun mRNA after 60 min of PMA treatment was used as the reference to study curcumin inhibitory activity.

Curcumin at 10, 15, or 20 μM together with PMA at 50 ng/ml were used to treat quiescent cells for 60 min. Fig. 1*b* shows that the inhibitory effect of curcumin correlates directly with curcumin dose. Dot hybridization further indicated that 10, 15, or 20 μM of curcumin inhibited the PMA-induced increase of c-jun mRNA by 21, 28, or 56%, respectively (Fig. 1*d*). We also analyzed the effect of curcumin on c-*fos* gene. The product of c-*fos* gene is thought to be complexed and synergistic with c-Jun/AP-1 (5). However, in contrast to c-jun, c-fos mRNA was not altered by curcumin at 30 min after PMA treatment (Fig. 1*c*). When the quiescent cells were treated with curcumin without PMA, a basal level of the c-jun mRNA (control) was seen (data not shown).

Curcumin Suppresses the PMA-Induced TRE-Binding Activity in Nuclear Extract. The product of c-*jun* gene, c-Jun/AP-1, is a transcriptional factor that functions by binding with a specific enhancer element TRE. To evaluate the curcumin effect on c-Jun/AP-1 protein, we examined the changes of TRE-binding activity in nuclear extract by using gel-retardation assays. After PMA treatment for 60 min with or without curcumin, crude nuclear extracts were isolated and treated with a synthetic oligodeoxynucleotide containing a TRE site. Fig. 2*a* shows that a major mobility band was inducible by PMA. Because this band nearly disappeared when the nuclear extract was preincubated with anti-c-Jun/AP-1 antibody, we attribute this band mostly to c-Jun/AP-1 protein. We found that, whatever 0.5 or 1 μg of poly(dI-dC) was added, curcumin (20 μM) could inhibit >50% of the PMA-induced TRE-binding activity.

To confirm the suppressive effect of curcumin on TRE-binding activity, a time-course experiment was done. NIH 3T3 cells were treated with PMA with or without 15 μM curcumin for 30, 60, 120, and 180 min. Nuclear extracts were isolated, and TRE-binding activity was assayed. The autoradiograph is shown in Fig. 2*b*; each band corresponding to DNA–protein complexes was cut from the gel, and radioactivity was quantitated by liquid scintillation counting (Fig. 2*d*). The data indicate a 3.8-fold increase of TRE-binding activity shortly after 30-min PMA treatment. This immediate induction was cycloheximide-resistant (Fig. 2 *c* and *d*), suggesting that the induction was via a posttranslational modification (3, 6). Relative TRE-binding activity then decreased to 3.0-fold at 60 min and increased again, reaching a maximum of 4.1-fold at 120 min. This two-phase kinetic profile was also seen in a separate experiment of another nuclear extraction. However, when NIH 3T3 cells were treated with PMA plus curcumin, the PMA induction of TRE-binding activity was clearly prevented (Fig. 2 *b* and *d*). Further experiments showed that curcumin could inhibit cycloheximide-resistant TRE-binding activity (Fig. 2 *c* and *d*). The result suggests that PMA-induced posttranslational modification of c-Jun/AP-1 was inhibited by curcumin. Besides TRE oligodeoxynucleotides, a TRE-containing *Hin*dIII–*Sph* I fragment of SV40 promoter enhancer region was also used as probe in gel-retardation assay. Similar results

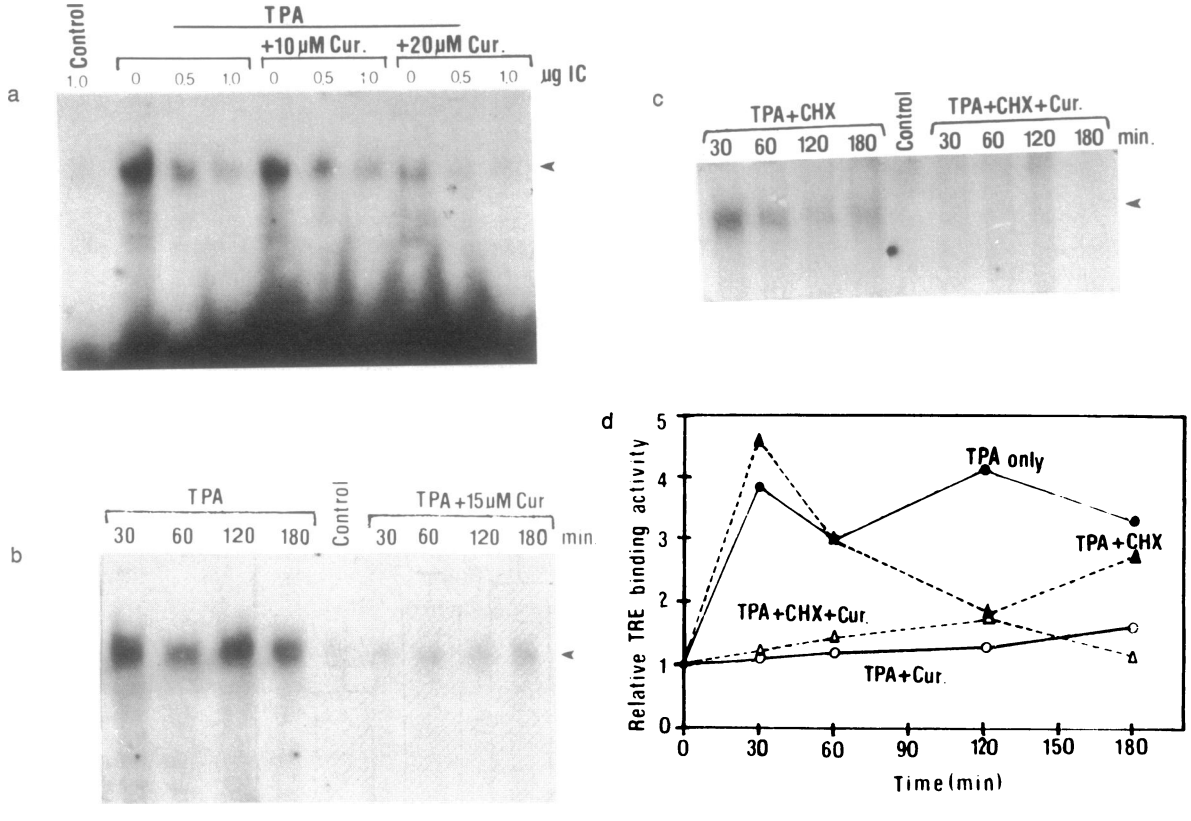

FIG. 2. Gel-retardation assay for different nuclear extracts. (*a*) Nuclear extracts were isolated from quiescent cells treated with PMA (TPA) with or without curcumin (Cur.) at 10 or 20 μM. Two micrograms of extract was mixed with a ^{32}P-labeled double-stranded oligodeoxynucleotide containing a TRE site. Different amounts (0, 0.5, or 1 μg) of poly(dI-dC) (IC) were also added as indicated. The entire reaction mixture was incubated at 30°C for 15 min, and then electrophoresis was done. After autoradiography, each band corresponding to DNA–protein complex was excised from the dried gel, and radioactivity was measured by liquid scintillation counting. Arrow, TRE/AP-1 complex. (*b*) Nuclear extracts were isolated from quiescent cells treated with PMA (TPA) with or without 15 μM curcumin (Cur.) for 30, 60, 120, and 180 min. The entire binding reaction mixture included 2 μg of nuclear extract, 0.5 ng of TRE probe, and 0.5 μg of poly(dI-dC). Arrow, TRE/AP-1 complex. (*c*) Nuclear extracts were isolated from quiescent cells treated as for *b* but with cycloheximide (CHX) at 30 μg/ml. Arrow, TRE/AP-1 complex. (*d*) Relative TRE-binding activity from experiments of *b* and *c*. The TRE-binding activity of control was designated as 1. Representative results from two independent experiments, at least, are shown.

were obtained to that of TRE oligodeoxynucleotides (data not shown). Because a TRE site also exists in the c-*jun* promoter, c-*jun* gene expression can be directly stimulated by its own activated preexisting c-Jun/AP-1 protein (6). We have described above that curcumin reduced PMA-induced c-jun mRNA; the decreased c-jun mRNA seemed to be from attenuation of PMA-induced TRE-binding activity.

Curcumin Represses SV40–CAT Gene Expression. We, moreover, explored whether curcumin could repress other c-Jun/AP-1-responsive gene expression. We tried to use a transient-expression assay to determine the effect of curcumin on PMA-induced transcriptional-enhancing activity of SV40-CAT (pSV2CAT) reporter gene. pSV2CAT contains the SV40 promoter enhancer region located upstream of the CAT gene (3, 4). The c-Jun/AP-1 activation was indicated as important for conferring inducibility by PMA upon this promoter enhancer region. As expected, 12-hr PMA induction of NIH 3T3 cells transfected with pSV2CAT plasmids enhanced the level of CAT expression 3- to 4-fold relative to uninduced cells (Fig. 3). However, the PMA-enhanced CAT level was dramatically abolished when transfected cells were treated with PMA plus 10 μM curcumin. The result showed the inhibitory effect of curcumin on PMA-induced transactivating activity of c-Jun/AP-1.

DISCUSSION

Both posttranslational and transcriptional events have accounted for the response of c-Jun/AP-1 and c-*jun* gene to PMA. Additional termination mechanisms are thought necessary in cells; otherwise, autoregulation of c-*jun* expression would continuously overproduce c-Jun/AP-1, which could be cytotoxic. Most recently, *jun*-B, another member of the AP-1 family (18), has been shown to inhibit the transactivating and transforming activities of c-*jun*, presumably by competition for binding to a single TRE site (19–21). In addition, DNA binding of Jun/Fos complex could be modulated by redox regulation of a single conserved cysteine residue located in the DNA-binding domains (22). In this study, we show that curcumin has an inhibitory effect on PMA-induced c-Jun/AP-1 activity. Curcumin itself is nonmutagenic and antimutagenic (ref. 23; unpublished data), and recently curcumin was reported (11) to possess an efficient inhibitory effect on PMA-induced tumor promotion on mouse skin. We propose that c-Jun/AP-1 may be a target of this blocking process because c-Jun/AP-1 is considered a key component of cellular transcriptional machinery for mediating changes in the transcription activity induced by PMA (2–7).

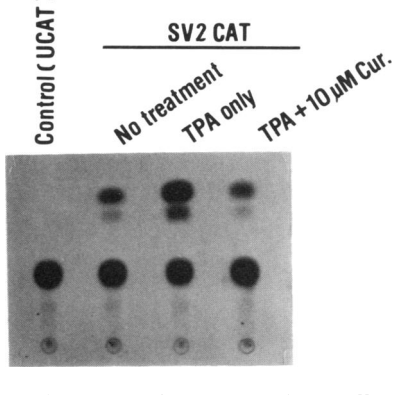

FIG. 3. Transient expression assay to detect effect of curcumin (Cur.) on PMA (TPA)-induced c-Jun/AP-1 trans-activation activity. NIH 3T3 cells were transfected with plasmids bearing the CAT gene under control of SV40 promoter enhancer region (SV2CAT) or plasmids lacking this control region (UCAT). Transfected cells were serum-starved for 36 hr and treated as indicated for 12 hr. CAT assays were done as described in text.

In considering PMA as a protein kinase C activator, the PMA-induced c-Jun/AP-1 DNA-binding activity may simply result from phosphorylation of c-Jun/AP-1. However, we cannot detect any significant increase of c-Jun/AP-1 phosphorylation after PMA treatment (unpublished data). On the other hand, the level of c-Fos phosphorylation rapidly increases in PMA-treated cells or upon serum stimulation (24). Moreover, c-Fos is detected as a complex with c-Jun/AP-1 in the stimulation of c-Jun/AP-1 TRE-binding activity after PMA treatment (5). We described above that PMA-induced c-jun but not c-fos mRNA level was decreased by curcumin and that this inhibition was probably from suppression of TRE-binding activity. Although the c-*fos* promoter contains TRE sites, these sites were believed to play some roles in rapid down-regulation of c-*fos* expression (25, 26). This mechanism could account for lack of any inhibition of c-fos mRNA by curcumin. However, curcumin still could have some effect on c-Fos protein and interfere with Jun–Fos complex formation.

While this paper was in preparation, some related observations were reported. Glucocorticoid hormones can inhibit collagenase gene expression induced by tumor promoter or inflammatory agents (8–10). The mechanism of interference is from repression of AP-1 activity by the glucocorticoid receptor. Protein cross-linking and coimmunoprecipitation experiments have indicated direct interaction between the glucocorticoid receptor and either c-Jun or c-Fos. Furthermore, adenovirus E1a represses transcription of the collagenase gene via the PMA-responsive element (27). E1a inhibits the trans-activating function of transcription factor AP-1 without reducing its synthesis and without any apparent change in DNA binding. Whether a curcumin-responsive receptor also exists or curcumin can activate some cellular protein(s) and then interact with c-Jun/AP-1 is unclear; the mechanism(s) of repression of AP-1 activity by curcumin is as yet unresolved.

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